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Long-term preservation of Potato leafroll virus, Potato virus S and Potato spindle tuber viroid in cryopreserved shoot tips

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Long-term preservation of Potato leafroll virus, Potato virus S and Potato spindle tuber viroid in cryopreserved shoot tips

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Running title: Cryopreservation of viruses and viroids

Abstract

Availability of and easy access to diverse viruses and viroids are a prerequisite in applied and basic studies related with virus and viroids. Plant viruses and viroids are obligate intracellular parasites that colonize only inside the living cells of the hosts, and long-term preservation of the virus and viroids is difficult. A protocol was described for long-term preservation of Potato leafroll virus, Potato virus S and Potato spindle tuber viroids in cryopreserved shoot tips of potato. Shoot regrowth levels following cryopreservation were higher (58-60%) in 1.5 mm-shoot tips than those (30-38%) in 0.5 mm-ones. All shoots recovered from 0.5 mm-shoot tips were PVS- and PSTVd-preserved, but none of them were PLRV-preserved. Cryopreservation of 1.5 mm-shoot tips resulted in 35%, and 100% of PLRV-, and PVS- and PSTVd-preserved shoots. Studies on cell survival patters and virus localization provided explanations to the varying PLRV-preservation frequencies produced by cryopreservation of the two sizes of shoot tips. Although micropropagation efficiencies were low during after 4 times (12 weeks) of subculture following cryopreservation, similar efficiencies were obtained after 6 times (16 weeks) of subculture in pathogen-preserved shoots recovered from cryopreservation, compared with the diseased *in vitro* stock shoots (the control). Similar patterns of the concentrations of the three pathogens-preserved shoots by RT-qPCR were similar to those of shoot micropropagation. The three pathogens cryopreserved in shoot tips were readily transmitted by grafting and mechanical inoculation to the potato hosts. PLRV, PVS and PSTVD represent a diverse range of plant viruses and viroids in terms of taxonomy and infectious ability. Therefore, shoot tip cryopreservation opens a new avenue for long-term preservation of the virus and viroids.

Keywords: cryopreservation; potato; shoot tips; virus, viroids

Introduction

Serological methods have long been used for plant virus detection (Hull, 2002). Antigen preparation is necessary in virus detection by serological methods.

Pathogen-derived resistance (PDR) has been used in genetic transformation to produce virus-resistant plants (Sudarshana et al., 2007). In the past several decades, plant-based production of vaccines provided a new strategy for the manufacture of vaccines for the prevention and treatment of human diseases (Salazar-González et al., 2015; Loh et al., 2017). Recent studies have shown plant viruses have potential applications to nanotechnology to produce nanodrugs (Lomonossoff and Evans, 2011; Yang et al., 2018). Availability of and easy access to diverse viruses is prerequisite in these applied studies and basic researches such as origin and evolution of viruses and viroids (Hull, 2002; Di Serio et al., 2017).

Plant viruses and viroids are obligate intracellular parasites that replicate only inside the living cells of the hosts by using the host's biochemical machinery (Hull, 2002; Flores et al., 2017). Since viruses and viroids do not capture or store free energy and therefore cannot live without living tissues (Hull, 2002; Flores et al., 2017). Preservation of viruses and viroids has long received interests of scientists working on virus- and viroids-related fields. So far, no information is available on long-term preservation of viroids. Over the several past decades, various strategies have been developed for preservation of plant viruses, including freeze (Fukumoto and Tochinara, 1998), freeze-drying (Hollings and Stone, 1970; Purcifull, 1975; Fukumoto and Tochinara, 1998; Yordanova et al., 2000), dehydration by physical drying (Grivell et al., 1971) and chemical drying (Mckinney et al., 1965), and *in vitro* culture (Chen et al., 2003; Infante et al., 2008), among which freeze-drying was the most widely and reliable method. With this method, although Cucumber mosaic virus could be preserved for up to 240 days, their infection efficiency rapidly decreased as preservation time increased, with only 7% infection frequency maintained after 240 day of preservation (Yordanova et al., 2000).

De and Suda-Bachmann (1979) reported Potato virus Y (PVY) and Watermelon mosaic virus (WMMV) contained in leaf powder could be cryopreserved in LN for 22 months for the former and 32 months for the latter, without any decreases in infectivity of the viruses. Recently, Fan et al. (2014) reported preservation of viral genomes in 700-y-old caribou feces from a subarctic ice patch. These data indicate

cryopreservation of virus seems a very promising long-term preservation method for plant viruses and viroids.

Cryopreservation, i.e. storage of living cells, tissues and organs in extra low temperatures, usually that of the liquid nitrogen (LN), is at present time considered an ideal means for long-term preservation of plant genetic resources. Since Sakai (1960) reported for the first time successful plant cryopreservation, this technique has been widely applied to almost all economically important agricultural crops (Wang et al., 2009a; Feng et al., 2011; Vollmer et al., 2017), horticultural plants (Höfera 2015; Wang et al., 2018a) and forest trees (Li et al., 2017). Recently, cryobanks have been established for some vegetatively propagated crops such as potato at International Potato Center (CIP) in Peru (Vollmer et al., 2017), apple at the Julius Kühn-Institute for Breeding Research on Fruit Crops in Germany (Höfer 2015) and garlic at National Agrobiodiversity Center in South Korea (Kim et al., 2012).

Shoot tip cryopreservation has been shown to efficiently eradicate plant pathogens including viruses (Wang and Valkonen, 2009a; Wang et al., 2009b; 2014a). Although shoot tip cryopreservation produced much higher frequencies of pathogen eradication than the traditional methods like meristem culture, pathogen eradication frequencies varied with types of pathogens and plants (Brison et al., 1997; Helliot et al., 2002; Wang et al., 2003, 2006; Li et al., 2016), as well as infection status and combinations of viruses and hosts (Wang and Valkonen, 2008; Li et al., 2016; Kushnarenko et al., 2017). Frequently, not all plants recovered from shoot tip cryopreservation were pathogen-free and a certain proportions of the recovered plants were still pathogen-infected, i.e. pathogen-preserved (Wang et al., 2009b, 2014a). Furthermore, shoot tip cryopreservation completely failed to eradicate viruses and viroids that can infect meristematic cells of the shoot tips, such as Raspberry bushy dwarf virus (RBDV, Wang et al., 2008), Apple stem grooving virus (ASGV, Li et al., 2016), Potato spindle tuber viroids (PSTVd, Bai et al., 2012) and Chrysanthemum stunt viroids (CSVd, Zhang et al., 2014). These data indicate that shoot tip cryopreservation may be used for long-term preservation of the viruses and viroids.

The present study attempted to cryopreserve Potato leafroll virus (PLRV),

Potato virus S (PVS) and PSTVd, three major pathogens attacking potato and widely present in potato-growing regions of the world, in shoot tips of potato 'Zihuabai'. Concentrations of the cryopreserved pathogens in the pathogen-preserved shoots were quantitatively analyzed by RT-pPCR. The cryopreserved pathogens were tested for their infectious ability to infect the healthy potato hosts by grafting and mechanical inoculation.

Results

Effects of size of shoot tips on shoot regrowth and pathogen preservation following cryopreservation

Sizes of shoot tips significantly affected shoot regrowth levels in cryopreserved shoot tips. Larger shoot tips (1.5 mm) produced significantly higher shoot regrowth levels (52-60%) than small ones (0.5 mm, 30-38%) among the three pathogen-infected shoots (Table 2). No significant differences were found in shoot regrowth levels produced in the same sizes of shoot tips of shoots infected with different pathogens (Table 1). For PLRV, cryopreservation of 0.5 mm shoot tips produced no virus-preserved shoots and 1.5 mm shoot tips resulted in production of 35% of virus-preserved shoots (Table 2). For PVS and PSTVd, 100% of shoots recovered from cryopreservation were pathogen-preserved, regardless of their sizes of shoot tips (Table 2).

Detection of PLRV, PVS and PSTVd by RT-PCR

At the beginning of the cryopreservation experiments, specific bands of 155 bp for PLRV, 137 bp for PVS and 224 bp for PSTVd were detected in all *in vitro* stock shoots infected with the corresponding pathogens, whereas no such bands were found in the healthy ones (Fig. 1A), thus ensuring sanitary status of the *in vitro* stock shoots used in this study. When RT-PCR was applied to detection of sanitary status in shoot recovered from cryopreservation after 6 times (18 weeks) of subculture, specific bands of about 155 bp for PLRV, 137 bp for PVS and 224 bp for SPTVd were detected in PLRV-, PSV and PSTVd-preserved shoots, respectively (Fig. 1A). For

PLRV, the results of virus detection were identical in shoots recovered from cryopreservation after 2 times (6 weeks) of post-culture and in plants grown in soil in the net-proof greenhouse for 3 months

Quantitative analysis by qRT-PCR of relative mRNA expression of PLRV, PVS and PSTVd in pathogen-preserved shoots recovered from cryopreservation

Stable and similar values were obtained in the virus- and viroid-infected shoots recovered from cryopreservation when the reference gene *EF-1α* was used (Table 3), indicating the RT-qPCR method used here was reliable. Patterns of relative mRNA expression levels of virus and viroid were similar in the three pathogen-preserved shoots recovered from cryopreservation and subcultured for different times (Table 3). The relative mRNA expression levels were low in the pathogen-preserved shoots after the 2 times (6 weeks) of subculture, significantly increased as subculture times increased and reached similar levels after 6 times (18 weeks) of subculture, compared with those of the *in vitro* diseased stock shoots without cryopreservation (Table 3).

Cell survival patterns

Living cells in the positive control showed dense TB-stained and well-preserved cytoplasm, and clearly visible nucleolus enclosed in the nucleus (Fig. 2A). Damaged or dead cells in the negative control showed reduced levels of TB-stained cytoplasm and the nuclei were heavily condensed (Fig. 2B). In cryopreserved shoot tips of PLRV-infected shoots, surviving cells were found in the upper part of apical dome (AD) (Fig. 2C) and leaf primordia (LPs) 1–3 (Fig. 2D-F). Surviving cells were occasionally found in LP 4 (Fig. 2G). Among 20 shoot tips tested, 6 shoot tips showed this survival patterns, accounting for 30%. Cells in LPs 5-6 (Fig. 2H and I), and other older tissues were damaged or killed.

Virus localization

With the histoimmunological virus localization protocol used in the present study, tissue infected with PLRV showed specific purple color reaction, while the healthy

tissue did not show such color reaction (Fig. 3A). PLRV was not detected in AD (Fig. 3B) and LPs 1-3 (Fig. 3B), while it was found in LP 4 and older tissues (Fig. 3B). Close reviews showed PLRV was phloem-limited (Fig. 3C and D).

Micropropagation of pathogen-preserved shoots recovered from cryopreservation

Patterns of micropropagation of the three pathogen-preserved shoots recovered from cryopreservation were similar (Table 4). Shoot length and number of nodes were significantly short and few in pathogen-preserved shoots recovered from cryopreservation after 4 times (12 weeks) of subculture, significantly increased as the subculture times increased and reached the similar levels after 6 times of subculture (18 weeks) of subculture, compared with pathogen-infected *in vitro* stock shoots (Table 4).

Detection by RT-PCR of PLRV, PVS and PSTVd in grafting and mechanically inoculated plants

Specific bands of 155 bp for PLRV, 137 bp for PVS and 224 bp for SPTVd were readily detected in the healthy rootstocks grafted with the corresponding pathogen-preserved scions after 4 weeks of grafting (Fig. 1B). The same was true in the healthy plants mechanically inoculated with cryopreserved PVS and PSTVd after 4 weeks of mechanical inoculation (Fig. 1C).

Discussion

Most of methods reported so far for preservation of plant viruses used dried materials (Mckinney et al., 1965; Grivell et al., 1971; Hollings and Stone, 1970; Purcifull, 1975; Fukumoto and Tochinara, 1998; Yordanova et al., 2000), and *in vitro* tissue culture preserved viruses in living tissue (Chen et al., 2003; Infante et al., 2008). When preserved in dried materials, some viruses were not stable and their infection ability decreased as time durations of preservation increased (Hollings and Stone, 1970; Grivell et al., 1971; Yordanova et al., 2000). For example, infection frequencies of

CMV preserved by freeze-drying were 95% and only 7% after 15 and 240 days of preservation, respectively (Yordanova et al., 2000). Following preservation, the virus can be transmitted only by mechanical inoculation to the target hosts (Mckinney et al., 1965; Hollings and Stone, 1970; Grivell et al., 1971; De and Suda-Bachmann, 1979; Yordanova et al., 2000). Since a number of plant viruses cannot be transmitted by mechanical inoculation (Hull, 2002), such preservation methods largely limited applications of the virus preservation. In *in vitro* culture for virus preservation, virus-infected tissues have to be periodically subcultured (Chen et al., 2003; Infante et al., 2008). Subculture has ricks of contamination, which may result in total loss of the stored materials. In addition, *in vitro culture* can be used only for medium-term virus preservation.

In the present study, PLRV, PVS and PSTVd were successfully cryopreserved in living shoot tips of potato. PLRV and PVS are a type number of the genus *Polerovirus* and *Potyvirus*, respectively, and attack a wide range of plant species (Valkonen, 2007). PSTVd belongs to the genus *Pospiviroid* and the family Pospiviroidae (Owens et al., 2012), and infects *Solanum* plants and a diverse array of ornamental species such as *Chrysanthmum* and *Argyranthemum* (Owens et al., 2017). PLRV is a phloem-limited virus and does not invade AD (Valkonen, 2007), which is also proven in the present study, while PSTVd is present in AD of plants (Zhang et al., 2015). PVS is a difficult-to-eradicate virus (Kushnarenko et al., 2017), indicating its infectious ability of shoot tips is stronger than PLVS. Thus, the three pathogens studied in the present study represent a wide range of viruses and viroids in terms of taxonomy and infectious ability. We previously reported successful preservation of ASGV in cryopreserved shoot tips of apple 'Gala' (Wang et al., 2018b). Gene sequencing of coat protein (CP) and movement protein (MP) of ASGV genome showed that cryopreserved ASGV shared 99.87% nucleotide identities with shoot tip culture-preserved virus, indicating cryopreserved virus is genetically stable. In addition, using the same potato cultivar and the cryogenic protocol, Wang et al. (2014b) reported no polymorphic bands were detected by genetic markers in the

plants recovered from cryopreservation, indicating the plants recovered from

cryopreservation were genetically stable, as already proven in many plant species (Wang et al., 2014a, 2018a; Li et al., 2017).

In this study, shoot tip cryopreservation produced 100% of PVS- and PSTVd-preserved plants, regardless of the shoot tip sizes. However, cryopreservation of 0.5 mm-shoot tips completely failed to preserve PLRV, and of 1.5-mm shoot tips resulted in 35% of virus-preserved plants and 65% of virus-free plants. In order to understand why frequencies of PLRV preservation varied with sizes of shoot tips, cell survival patterns and virus localization were conducted. Results showed PLVR was not present in AD and LPs 1-3, but it was readily found in LP 4 and older tissues. The majority of cells in AD and some cells in LPs 1-3 survived following cryopreservation. A few cells in LP 4 survived in about 30% of cryopreserved shoot tips tested. This figure is almost equally to 35% of the virus-preserved shoots. All the data generated above provided explanations to varying frequencies of PLRV-preserved shoots recovered from cryopreservation when 0.5 mm- and 1.5 mm-shoot tips were used for cryopreservation.

When viruses and viroids are cryopreserved in shoot tips, cryopreservation durations of shoot tips, shoot regeneration of cryopreserved shoot tips and propagation efficiency of the recovered shoots are important factors determining pathogen preservation efficiency. Once samples are stored in LN, cellular divisions and metabolic processes are arrested, and theoretically, plant materials can be stored for a definitive period of time (Benson, 2008). In plant preservation, a sample is considered as successfully cryopreserved if it has a minimum recovery rate of ≥30% (Vollmer et al., 2017). Potato is a plant that is more vulnerable to cryopreservation, and shoot regrowth levels were generally high (>50%) in most of the previous studies (Wang et al., 2009a; Vollmer et al., 2017). Shoot regrowth levels of 58-60% were obtained in the present study, which can be considered high enough for cryopreservation (Vollmer et al., 2017). Previous studies showed that shoot regrowth levels maintained unchanged in potato shoot tips that had been cryopreserved for up to 10 years (Keller et al., 2006). In the present stud, although shoot proliferation levels of the pathogen-cryopreserved shoots were lower than that of the control after 4 times

(12 weeks) times of subculture, they reached similar levels as the control after 6 times (18 weeks) time of subculture. These data demonstrate pathogen-preserved shoots recovered from cryopreservation can be efficiently micropropagated. In addition, infectious abilities of virus- and viroids-preserved plants recovered from cryopreservation were verified by grafting and mechanical inoculation to the potato hosts.

In conclusion, PLRV, PVS and PSTVd were for the first time successfully cryopreserved in shoot tips. These pathogens represent a diverse range of viruses and viroids in term of the infectious ability and taxonomy. Shoot tip cryopreservation opens a new avenue for long-term preservation of viruses and viroids, and has potential applications to studies in fields related.

Materials and methods

Plant materials

Potato 'Zihuabai', a cultivar susceptible to the pathogens studied, was used in the present study. *In vitro* certified healthy (virus-free) shoots and diseased shoots single-infected with PLRV, PVS and PSTVd, respectively, were maintained on a basic medium (BM) composed of solid half-strength Murashige and Skoog (1962) medium (MS) supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar (pH=5.8), according to Li et al. (2013, 2018). Sanitary status of all the *in vitro* stock shoots was confirmed before cryopreservation using reverse transcription-polymerase chain reaction (RT-PCR), as described below. The cultures were grown at 22±2 C under a 16-h photoperiod at 50 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes. Subculturing was conducted every 3 weeks.

Virus cryopreservation

Shoot tips were cryopreserved by droplet-vitrification, as described by Wang et al

(2013, 2014b). Nodal segments (1 cm in length), each containing an axillary bud, were taken from 3-weeks old shoots of ock cultures and cultured on BM under the same conditions as described for the *in vitro* stock cultures. Shoots (1–1.5 cm in length)

developed from axillary buds after 7 days of culture and transferred to a growth chamber for cold-hardening in the dark at 5 °C for three weeks. Two sizes of shoot tips: 0.5 mm and 1.5 mm in length containing 2-3 and 5-6 leaf primordia (LPs), respectively, were excised from the cold-hardened stock shoots and precultured on BM containing 0.3 M sucrose in the dark at 5 °C for 3 days. Precultured shoot tips were treated for 30 min with a loading solution containing 2 M glycerol and 0.4 M sucrose in MS medium and then dehydrated with PVS2 (Sakai et al., 1990) at 0 °C for 40 min. PVS2 consisted of 30% (w/v) glycerol, 15% (w/v) dimethyl sulfoxide (DMSO), 15% (w/v) ethylene glycol and 0.4 M sucrose in MS medium. Dehydrated shoot tips were transferred onto 3 µL PVS2 droplets on aluminum foils, followed by a direct immersion in LN for 1 h. Frozen foil strips with shoot tips were removed out from LN and rapidly transferred into an unloading solution composed of MS supplemented with 1.2 M sucrose at 25 °C for 20 min.

Post-culture for shoot recovery of cryopreserved shoot tips

Cryopreserved shoot tips were post-cultured on a shoot recovery medium composed of BM supplemented with 0.05 mg L^{-1} GA_3, for shoot regrowth. The cultures were grown kept in the dark at 22 ± 2 °C for 3 days and then transferred into the light condition, as used for the *in vitro* stock shoots. Shoot regrowth was defined as percentage of the total number of shoot tips regenerating into normal shoots (≥5 mm) 6 weeks after post-culture. Subculturing was conducted every 3 weeks.

Detection of PLRV, PVS and PSTVd by RT-PCR

Detection of PLRV, PVS and PSTVd was conducted in the *in vitro* stock shoots before cryopreservation, to confirm their sanitary status. Detection of PLRV, PVS and PSTVd was conducted again in shoots recovered from cryopreservation after 2 times (6 weeks) of post-culture. For PVS and PSTVd, since all samples tested this time were positive responses, and they were considered to be pathogen-preserved and used for micropropagation. For PLRV, samples showing positive responses were considered to be virus-preserved and used for micropropagation. Samples showing

negative responses were considered to be virus-free and cultured for further growth. After 6 times (18 weeks) of post-culture, they were transferred to soil in the net-proof greenhouse condition. Their virus status was tested again after 3 months of growth.

Total RNA was extracted from shoots with leaves (0.5 g) using the Trizol Reagent (Invitrogen Ltd., Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was synthesized, according to Li et al. (2013; 2018). The primers used for the three pathogens were listed in Table 1. The PCR reaction was performed as described by Li et al. (2013, 2018). Programs used for PLRV, PVS and PSTVd were as followings: for PLRV: initial denaturation step at 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 56 °C for 20 s and 72 °C for 30 s, followed by the final extension step at 72 °C for 10 min. The PCR products were separated by electrophoresis in 1.5 % agarose gel in Tris-acetate (TAE) buffer (40 mM Tris-acetate, 1 m MEDTA, pH, 8.0), stained with ethidium bromide, and visualized and photographed under ultraviolet light.

Quantitative analysis by RT-qPCR of relative mRNA expression levels of pathogens in pathogen-preserved shoots recovered from cryopreservation

Relative mRNA expression levels of the pathogens were quantitatively analyzed by RT-qPCR in the pathogen-preserved shoots recovered from cryopreservation during the 2 times (6 weeks) to 6 times (18 weeks) of subculture. Total RNA extraction and cDNA reverse transcription was described as above. The RT-qPCR was performed using a CFX1000 (Bio-Rad, USA) instrument and a SYBR Premix ExTaq II Kit (Takara, Dalian, China) reagent. EF1a was used as reference gene (Wang et al. 2018b). All primers and the reference gene used for RT-qPCR are listed in Table 1. The relative expression levels of each gene were normalized to the expression of *EF1a* gene (Wang et al. 2018b). The relative mRNA expression levels of PLRV, PVS and PSTVd were expressed as Ct values (Chung et al., 2016).

Histological observations on cell

In order to understand why frequencies of PLRV cryopreservation varied with different sizes of shoot tips, cell survival patterns in cryopreserved shoot tips and virus localization in the PLRV-infected *in vitro* stock shoots were conducted, according to Wang et al. (2014b) and Li et al (2016), respectively. For histological observations on cell survival patterns, cryopreserved shoot tips of PLRV-infected shoots were collected 1 day after post-culture, fixed in formalin-acetic-alcohol (FAA) (ethanol : formalin : acetic acid = 18:1:1) for 24 h, and dehydrated through an incremental ethanol series (70, 85, 90, 95, and 100% ethanol). After embedding in paraffin, sections (5 μm thick) were cut with a microtome (Leica 2235, Germany) and stained with 0.01% toluidine blue (TB) (Sakai, 1973). The stained sections were observed under a light microscope (Leica DM2000, Germany). Shoot-tips that were freshly excised from stock shoots served as a positive control, while those that were freshly excised, directly immersed in LN served as a negative control. Both positive and negative controls received the same histological processes as described above.

For PLRV localization, shoot tips were harvested from the *in vitro* PLRV-infected stock shoots. Samples of the healthy *in vitro* shoots were used as negative controls. Cross sections were obtained, as described above. Virus localization was conducted, as described by Li et al. (2016). The sections were treated with phosphate buffered saline (PBS) containing 4% bovine serum albumin (BSA) for 30 min, followed by overnight incubation at 5◦C with coat protein (rabbit polyclonal antibodies to PLRV) (dilution 1:500 with PBS). After washing with PBS three times, the samples were incubated with alkaline phosphatase-conjugated antibodies (mouse anti-rabbit monoclonal antibodies) (dilution 1:500 with PBS) for 30 min at room temperature. After washing again three times with PBS, samples were stained using a freshly prepared Fuchsin substrate solution. The sections were observed with a light microscope (Leica DM 2235).

Micropropagation of pathogen-infected shoots recovered from cryopreservation

After the 2 times (6 weeks) of post-culture for shoot regrowth, shoots recovered from cryopreservation were transferred onto BM and cultured under the same light

conditions as used for in vitro stock shoots, for micropropagation. Pathogen-infected *in vitro* shoots without cryopreservation were used as controls. Subculturing was conducted every 6 weeks. Shoot length and node number, the two major parameters determining micropropagation efficiency in potato, were recorded every 2 times of subculture.

Establishment of the diseased plants recovered from cryopreservation in soil

After 6 times (18 weeks) of subculture, plantlets with well-developed roots were transferred into soil and grown in the net-proof house, with regular irrigation and fertilization, according to practical managements. The plants were used for the pathogen transmission by grafting and mechanical inoculation, as described below.

Transmission of the cryopreserved pathogens to potato hosts by grafting and mechanical inoculation

For grafting transmission, PLRV-, PVS- and PSTVd-preserved plants that recovered from cryopreservation and had grown for 6 weeks in soil in net-proof greenhouse were used as inocula materials. The healthy plants of potato 'Zihuabai' grown in the same net-proof greenhouse were used as rootstocks. Shoot segments (2.0-2.5 cm), each containing 2-3 well-developed leaves, were excised from middle to low parts of the pathogen-preserved plants and used as scions. A 'V' shape (approximately 0.5 cm in length) was cut at the base of the scions. The healthy rootstocks were decapitated approximately 5.0 cm above the soil. A vertical cut (approximately 0.6 cm in length) was made at the top of the rootstocks. Grafting was performed by inserting the 'V' shape of scions into the vertical cut of rootstocks, and then parafilm was used to fix the graft union. Sanitary status of the rootstocks was tested by RT-PCR after 4 weeks of grafting, as described above.

For mechanical transmission, since PLRV cannot be mechanically transmitted, it was excluded in this experiment. PVS- and PSTVd-preserved plants that recovered from cryopreservation and had been grown in soil in net-proof greenhouse for 3

months were used for mechanical inoculation to transmit PVS and PSTVd to potato

'Zihuabai' and *Solanum jasminoides* plants, respectively. All plants were grown in a net-proof greenhouse at 22±2 ºC with 16-h photoperiod. Mechanical transmission of PVS was conducted as described by Li et al. (2015). Briefly, leaves positioning between 5 to 7 nodes (counting from shoot terminal downward) were taken from PVS-preserved plants and ground with PBS (1 g/5 ml) contained in a plastic bag (Li et al., 2015). The freshly prepared virus inoculum was maintained on ice until use. The first 2-3 fully-opened leaves (counting from shoot terminal downward) of the inoculum plants that had been slightly dusted with carborundum were inoculated by rubbing gently with a cotton-swab soaked in the virus solution. Plants inoculated with the inoculation buffer served as negative controls. Inoculated plants were grown in a growth chamber at 25 C under the light conditions. Leaves were taken from the inoculated plants after 4 weeks of inoculation and used for virus detection by RT-PCR, as described above.

Mechanical transformation of SPTVd was performed, according to Verhoeven et al. (2010). Briefly, leaves positioning between 5 to 7 nodes (counting from shoot terminal downward) were taken from PSTVd-preserved plants and ground with SPB (1 g/10 ml), as described above. The freshly prepared viroid inocula were maintained on ice until use. The first 2-3 fully-opened leaves (counting from shoot terminal downward) of the inoculum plants were inoculated with PSTVd-contaminated razor blades. Plants inoculated with PBS served as negative controls. Inoculated plants were grown in a growth chamber at 25 ºC under the light conditions. Leaves were taken from the inoculated plants after 4 weeks of inoculation and used for viroid detection by RT-PCR, as described above.

Experimental design and data analysis

For experiments of shoot tip cryopreservation and micropropagation, ten samples were included in each treatment of three replicates. All experiments were conducted twice. Data were presented as means with their standard errors and analyzed using one-directional ANOVA and Students' t-test. Significant differences were calculated at

P<0.05. Twenty samples were included in histological observations on cell survival patterns, PLRV localization, and virus transmission by grafting and mechanical inoculation. Five biological replicates were used in analyses of RT-PCR and RT-qPCR.

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Author contribution statements:

J-W Li, M-R Wang and L Zhao: performance of experiments, data collection and analysis, and preparation of manuscript; H-Y Chen: assistance to performance of experiments; Z-H Cui: assistance to data collection and analysis; Z Zhang: valuable discussions; D-R Blystad: valuable discussions; Q-C Wang: chief scientist of the project, financial supports; experimental design and preparation of manuscript.

Competing interests

The authors declare no competing interests.

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Table 1 Names, sequences and amplified bands of primers and reference used for detection of PLRV, PVS and PSTVd by RT-PCR and real time RT-qPCR in potato 'Zihuabai'.

Primer name	Sequence(5'-3')	Amplified bands (bp)	Ref
PLRV-F	CCCACTGGAAGAGGGATGTAACT	155	Designed in this study
PLRV-R	CTTCGGATGCTTCCCGCTCTA		
PVS-F	CAGATGTGCCCAGAGCCAAGT	137	Designed in this study
PVS-R	GCCAGACCCAGATTACCAAAA		
PSTVd-F	ATCGATGAGGAGCGCTTCAGGGATC	224	Designed in this study
PSTVd-R	GTCGACGGAGCTTCAGTTGTTTCC		
*EF1a-F**	ATTGGAAACGGATATGCTCCA	101	Wang et al. 2018
EF1a-R	TCCTTACCTGAACGCCTGTCA		

**EF1a*-F and *EF1a*-R were used only in RT-qPCR.

Table 2 Effects of shoot tip sizes on shoot regrowth levels and frequencies of pathogen preservation in diseased *in vitro* shoots following cryopreservation in potato 'Zihuabai'

Virus infection status of stock shoots	Size of shoot tips	Shoot regrowth (%)	Virus-free shoots (%)	Virus-preserved shoots (%)
PLRV	0.5 mm, 2-3 LPs	35±5b	100 (20/20)	0 (0/20)
	1.5 mm, 5-6 LPs	60±5a	65 (13/20)	35 (7/20)
PVS	0.5 mm, 2-3 LPs	38±5b	0 (20/20)	100 (20/20)
	1.5 mm, 5-6 LPs	58±5a	0 (20/20)	100 (20/20)
PSTVd	0.5 mm, 2-3 LPs	30±5b	0 (20/20)	100 (20/20)
	1.5 mm, 5-6 LPs	52±5a	0 (20/20)	100 (20/20)

Size of shoot tips was defined as length (mm) + number of leaf primordium (LP).

Numbers in parentheses indicate positive reactions to PLRV, PVS and PSTVd/total samples tested by RT-PCR.

Data of shoot regrowth are presented as means ± SE and followed by different letters indicate significant differences at $P<0.05$ analyzed by Student's *t*-test.

Table 3 Relative mRNA expressions levels (Ct values) of PLRV, PVS and PSTVd analyzed by RT-qPCR in pathogen-preserved shoots recovered from cryopreservation after different times of subculture in potato 'Zihuabai'.

Types of shoots	Subculture times after shoot regrowth		
	3	6	9
PLRV			
Virus-preserved shoots	48.2±2.7Aa	36.1±1.8Ba	24.5±0.9Ca
Infected i*n vitro* stock shoots	23.1±1.1Ab	22.5±1.0Ab	22.3±0.9Aa
PVS			
Virus-preserved shoots	42.5±2.6Aa	30.3±1.5Ba	20.4±0.8Ca
Infected *in vitro* stock shoots	20.5±0.7Ab	20.8±0.9Ab	20.0±0.8Aa
PSTVd			
Viroid-preserved shoots	38.6±2.1Aa	28.4±1.8Ba	21.5±0.7Ca
Infected *in vitro* stock shoots	21.4±0.8Ab	20.5±0.7Ab	20.8±0.8Aa
Ct value of reference gene *EF-1α*	18.2±0.8	18.5±0.8	17.8±0.7

Results are presented as means ± SE. Data followed by upper-case letters in the same lines and by low-case letters in the same column of the same pathogen indicate significant differences at $P<0.05$ by Student's *t*-test.

Table 4 Micropropagation of pathogen-preserved shoots recovered from cryopreservation after different times of subculture in potato 'Zihuabai'.

Types of shoots	Subculture times after shoot regrowth					
	2		4		6	
	Shoot length (cm)	Node number	Shoot length (cm)	Node number	Shoot length (cm)	Node number
PLRV						
Virus-preserved shoots	0.7±0.2Cb	1.5±0.2Cb	3.6±0.4Bb	4.5±0.5Bb	6.8±0.4Aa	7.4±0.7Aa
Virus-infected *in vitro* stock shoots	6.9±0.4Aa	7.9±0.7Aa	7.1±0.6Aa	8.0±0.8Aa	7.4±0.5Aa	8.4±0.9Aa
PVS						
Virus-preserved shoots	0.6±0.2Cb	1.4±0.2Cb	3.4±0.4Bb	4.1±0.3Bb	6.5±0.5Aa	7.3±0.6Aa
Virus-infected *in vitro* stock shoots	6.6±0.5Aa	7.8±0.7Aa	7.2±0.7Aa	8.1±0.7Aa	7.0±0.5Aa	7.3±0.6Aa
PSTVd						
Viroid-preserved shoots	0.6±0.1Cb	1.4.0±0.2Cb	3.3±0.3Bb	4.0±0.4Bb	6.5±0.5Aa	7.1±0.6Aa
Viroid-infected *in vitro* stock shoots	6.5±0.5Aa	7.8±0.6Aa	7.1±0.8Aa	8.1±0.7Aa	7.0±0.5Aa	8.2±0.7Aa

Results are presented as means ± SE. Data followed by upper-case letters in the same lines of the same parameters and by low-case letters in the same column of the same pathogen indicate significant differences at $P<0.05$ by Student's *t*-test.

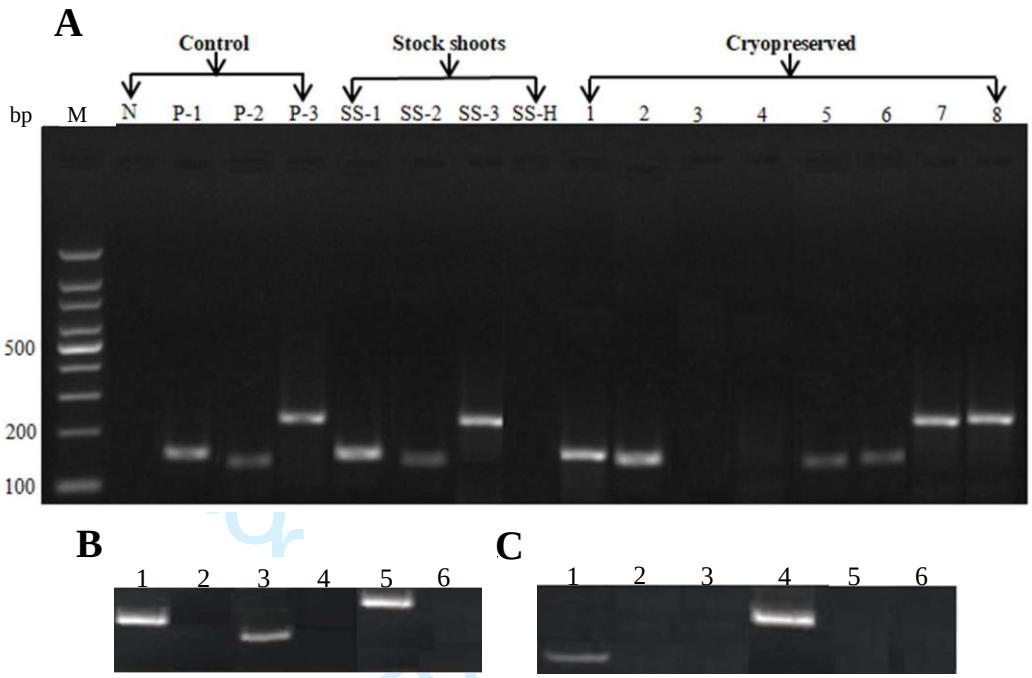


Figure 1 Detection by RT-PCR of potato leafroll virus (PLRV), Potato virus S (PVS) and Potato spindle tuber viroid in *in vitro* stock shoots before cryopreservation and shoots recovered after cryopreservation in potato 'Zihuabai' (A), in the healthy rootstocks grafted upon the virus- and virus-preserved rootstocks in potato 'Zihuabai' (B), and in the healthy potato 'Zihuabai' and *Solanum jasminoides* plants inoculated with PVS- and PSTVd-preserved shoots, respectively (C). (A): M=molecular marker; N=negative control; P1=positive control of PLRV, P2=positive control of PVS; P3=positive control of PSTVd; SS1=PLRV-infected stock shoots; SS2=PVS-infected stock shoots; SS3=PSTVd-infected stock shoots; SS-H=healthy stock shoots; Lanes 1-2=shoots recovered from cryopreserved shoots of PLRV-infected stock shoots; Lanes 3-4=shoots recovered from cryopreserved shoots of PLRV-infected stock shoots; Lanes 5-6=shoots recovered from cryopreserved shoots of PVS-infected stock shoots; Lanes 7-8=shoots recovered from cryopreserved shoots of PSTVd-infected stock shoots. (B): lane 1=grafted with PLRV-preserved scions; lane 2=grafted with the healthy scions; lane 3= grafted with PVS-preserved scions; lane 4=grafted with the healthy scions; lane 5=grafted with PSTVd-preserved scions; lane 6=grafted with the healthy scions.

(C): lane 1=inoculated with PVS-preserved samples; lane 2=mock inoculation for PVS;

hy samples; lane 4=inoculated with PSTVd-preserved samples; lane 5=mock inoculation for PSTVd; lane 6=inoculated with the healthy samples.

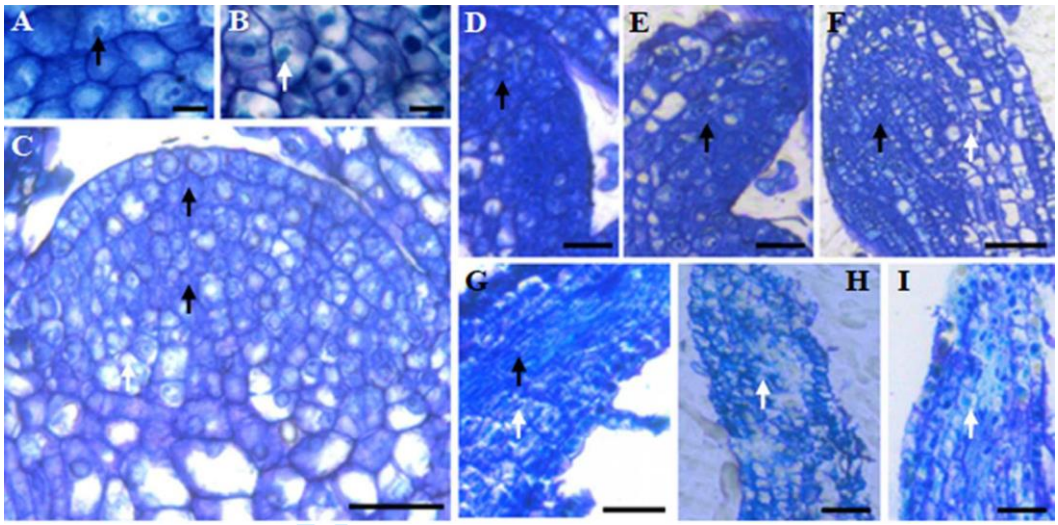
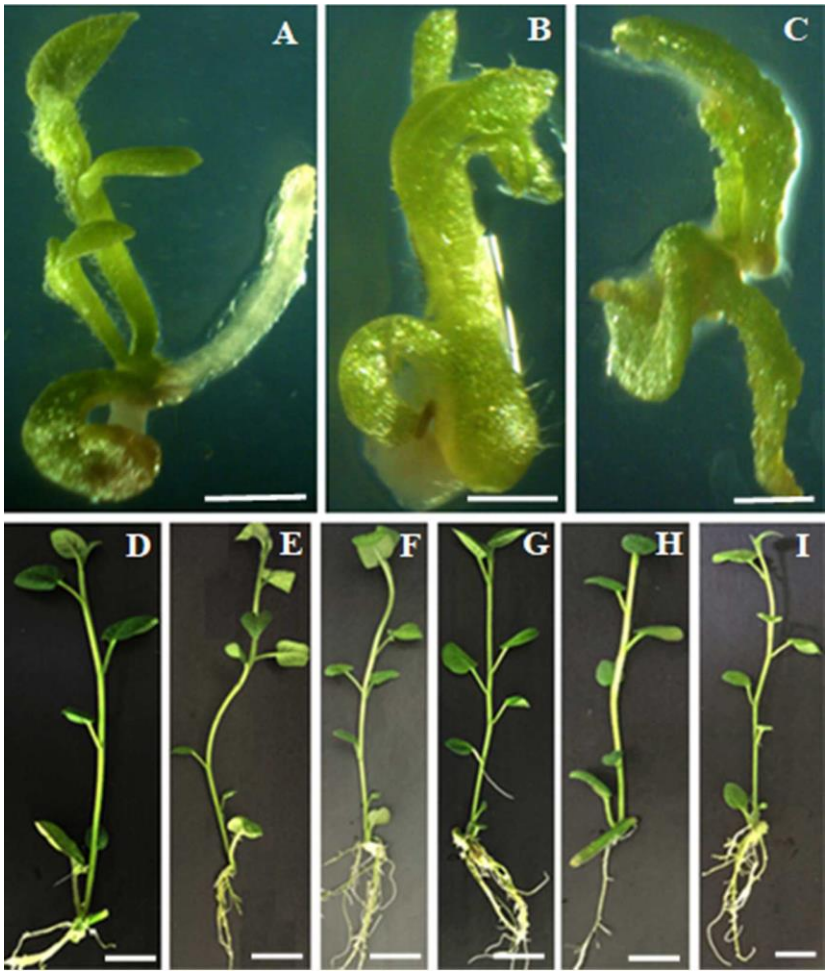


Figure 2 Cell survival patterns in cryopreserved shoot tips of PLRV-infected shoots of potato' Zihuabai'. Positve (A) and negative (B) control. Apical dome of cryopreserved shoot tips (C). Leaf primordia 1 (D), 2 (E), 3 (F), 4 (G), 5 (H) and 6 (I). Living cells and damage or dead cells are indicated by black arrows and white arrows, respectively. Scale bars in A and B=10 μm, and in C-I=20 μm

ropropagation of PLRV-, PVS- and PSTVd-cryopreserved shoots following cryopreservation in potato ‘Zihuabai’. Shoot regrowth from cryopreserved shoot tips of

Figure 3 Shoot regrowth and



PLRV (A)-, PVS (B)- and PSTVd (C)-infected shoots after the 2 times (6 weeks) of subculture. Micropropagated shoots from PLRV (D)-, PVS (F)- and PSTVd (H)-cryopreserved shoots after 6 times (18 weeks) of subculture. Micropropagated shoots from *in vitro* stock shoots infected with PLRV(E)-, PVS (G)- and PSTVd (I)-infected shoots after 9 times (27 weeks) of subculture. Scale bars in A, B and C=0.5 cm, and in D-I=1.0 cm.

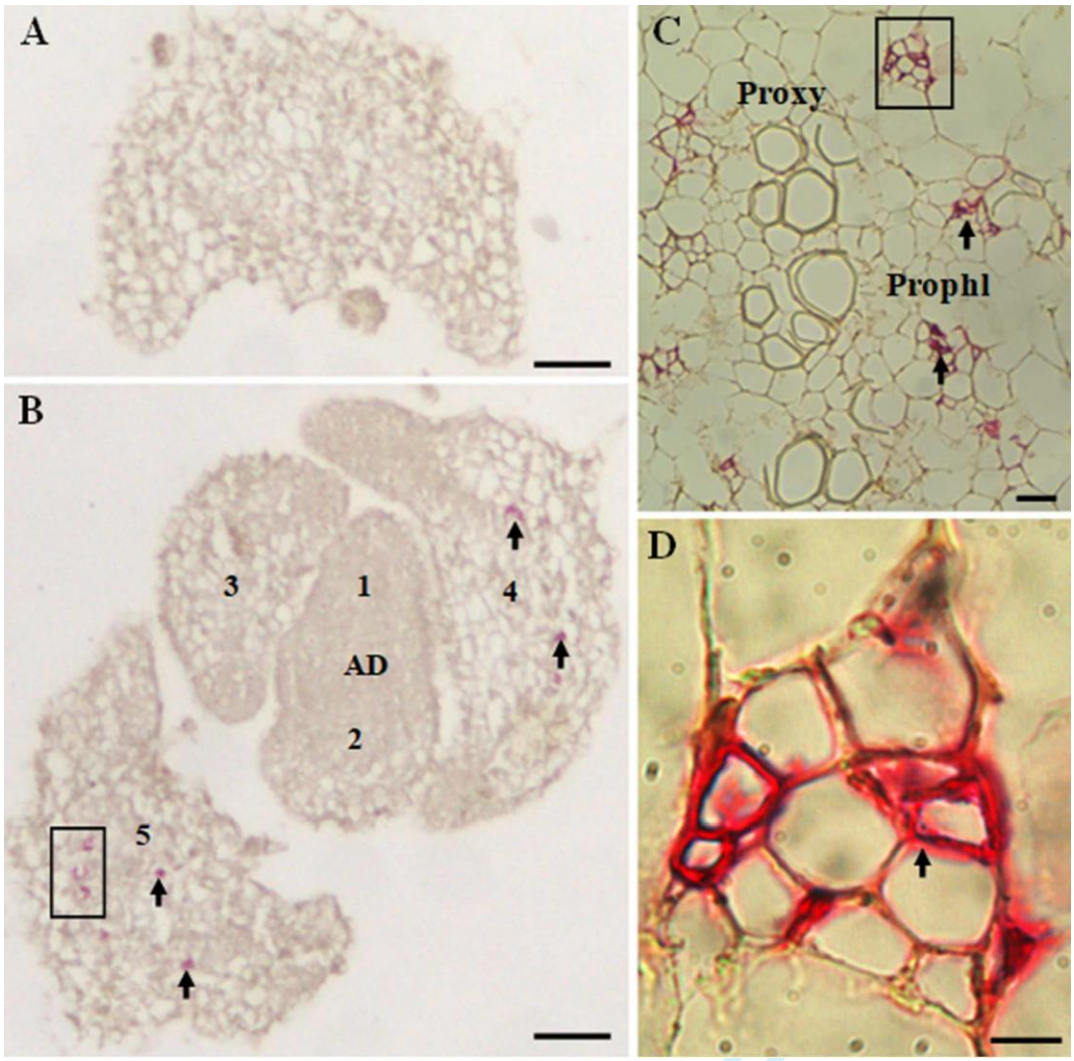


Fig 4 Histoimmunological localization of Potato leafroll virus (PLRV) in the diseased *in vitro* shoot tips of potato 'Zihuabai'. Cross section of healthy tissues (A) Cross section of virus-infected shoot tips (B). Close view of the black square in B (C). Close view of the black square in C (D). PLRV-infected tissues gave purple color reaction, as indicated by black arrows, while healthy tissues did not give such reaction. Virus AP=apical dome; 1, 2, 3, 4 and 5=leaf primordia 1, 2, 3, 4 and 5, respectively; Prophl=prophloem; Proxy=proxylem. Scale bars in A and B=50 µm, and in C and D= 10 µm.